PCT/US 97/01978

09/09/1605

6 DOE LABEL

U.S. PATENT APPLICATION

SERIAL NUMBER	FILING DATE	CLASS	GROUP ART UNIT
60/012,111 PROVISIONAL	02/06/96		

APPLICANT

ANNE R. MILLER, INDIANAPOLIS, IN.

REC'D 03 APR 1997
WIPO PCT

CONTINUING DATA********
VERIFIED

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FOREIGN/PCT APPLICATIONS********
VERIFIED

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PRIORITY DOCUMENT

FOREIGN FILING LICENSE GRANTED 04/15/96

STATE OR COUNTRY	SHEETS DRAWING	TOTAL CLAIMS	INDEPENDENT CLAIMS	FILING FEE RECEIVED	ATTORNEY DOCKET NO.
IN	D			$150.00	P-9872

ADDRESS

ELI LILLY AND COMPANY
PATENT DIVISION DC 1104
LILLY CORPORATE CENTER
INDIANAPOLIS IN 46285

TITLE

DIABETES THERAPY

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60012111

PATENT APPLICATION SERIAL NO. _____

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE
FEE RECORD SHEET

PTO-1556
(5/87)

Mo[redacted]16 (6-95)
Approved for use through 04/11/98. OMB 0651-0037
Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

60012111

PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53 (b)(2).

Docket Number	P-9872	Type a plus sign (+) inside this box -->	+

INVENTOR(s)/APPLICANT(s)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
Miller	Anne	Reifel	Indianapolis, Indiana

TITLE OF THE INVENTION (280 characters max)

DIABETES THERAPY

CORRESPONDENCE ADDRESS

Eli Lilly and Company
Patent Division/D.C. 1104
Lilly Corporate Center
Indianapolis, Indiana 46285

STATE	IN	ZIP CODE	46285	COUNTRY	USA

ENCLOSED APPLICATION PARTS *(check all that apply)*

X	Specification	Number of pages	21		Small Entity Statement
	Drawing(s)	Number of Sheets			Other (Specify)

METHOD OF PAYMENT *(check one)*

			PROVISIONAL FILING FEE AMOUNT ($)	
	A check or money order is enclosed to cover the Provisional filing fees			$150.00
X	The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number:	05-0830		

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

[X] No.

[ ] Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

SIGNATURE [signature] Date 02 / 06 / 96

TYPED or PRINTED NAME RONALD S. MACIAK

REGISTRATION NO. *(if appropriate)* 35,262

[ ] Additional inventors are being named on separately numbered sheets attached hereto

PROVISIONAL APPLICATION FILING ONLY

... US Date of Deposit Feb 6, 1996

... paper or fee is being deposited with the United States Postal Service ... under 37 CFR 1.10 on the date indicated above ... Commissioner of Patents and Trademarks, Washington, D.C. 20231

Ronald S. Maciak [signature]

DIABETES THERAPY

Technical Field of Invention

The present invention relates to genetic engineering as applied to pharmaceutical research and development in the general area of gene therapy. In particular, this invention relates to the use of stable, transformed human cell lines as vehicles for transferring functional human genes useful in the tratment of *diabetes mellitus*.

Background of the Invention

Endocrine secretions of pancreatic islets are regulated by complex control mechanisms driven not only by blood-borne metabolites such as glucose, amino acids, and catecholamines, but also by local paracrine influences. The major pancreatic islet hormones, glucagon, insulin and somatostatin, interact with specific pancreatic cell types (A, B, and D cells, respectively) to modulate the secretory response. Although insulin secretion is predominantly controlled by blood glucose levels, somatostatin inhibits glucose-mediated insulin secretion. In addition to inter-islet paracrine regulation of insulin secretion, there is evidence to support the existence of insulinotropic factors in the intestine. This concept originates from observations

that glucose taken orally is a much more potent stimulant of insulin secretion than is a comparable amount of glucose given intravenously.

The human hormone glucagon is a 29-amino acid hormone produced in pancreatic A-cells. The hormone belongs to a multi-gene family of structurally related peptides that include secretin, gastric inhibitory peptide, vasoactive intestinal peptide and glicentin. These peptides variously regulate carbohydrate metabolism, gastrointestinal mobility and secretory processing. However, the principal recognized actions of pancreatic glucagon are to promote hepatic glycogenolysis and gluconeogenesis, resulting in an elevation of blood sugar levels. In this regard, the actions of glucagon are counter regulatory to those of insulin and may contribute to the hyperglycemia that accompanies *diabetes mellitus* (Lund, P.K., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 79:345-349 (1982)).

When glucagon binds to its receptor on insulin producing cells, cAMP production increases which in turn stimulates insulin expression (Korman, L.Y., *et al.*, *Diabetes*, 34:717-722 (1985)). Moreover, high levels of insulin down-regulate glucagon synthesis by a feedback inhibition mechanism (Ganong, W.F., *Review of Medical Physiology*, Lange Publications, Los Altos, California, p. 273 (1979)). Thus, the expression of glucagon is carefully regulated by insulin, and ultimately by serum glucose levels.

Preproglucagon, the precursor form of glucagon, is encoded by a 360 base pair gene and is processed to form proglucagon (Lund, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 79:345-349 (1982)). Patzelt, *et al.* (*Nature*, 282:260-266 (1979)) demonstrated that proglucagon is further processed into glucagon and a second peptide. Later experiments demonstrated that proglucagon is cleaved carboxyl to Lys-Arg or Arg-Arg residues (Lund, P.K., *et al.*, Lopez L.C., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 80:5485-5489 (1983), and Bell, G.I., *et al.*, *Nature* 302:716-718 (1983)). Bell, G.I., *et al.*, also discovered that proglucagon contained three

discrete and highly homologous peptide regions which were designated glucagon, glucagon-like peptide 1 (GLP-1), and glucagon-like peptide 2 (GLP-2). Lopez, *et al.*, demonstrated that GLP-1 was a 37 amino acid peptide and that GLP-2 was a 34 amino acid peptide. Analogous studies on the structure of rat preproglucagon revealed a similar pattern of proteolytic cleavage at Lys-Arg or Arg-Arg residues, resulting in the formation of glucagon, GLP-1, and GLP-2 (Heinrich, G., *et al.*, *Endocrinol.*, 115:2176-2181 (1984)). Finally, human, rat, bovine, and hamster sequences of GLP-1 have been found to be identical (Ghiglione, M., *et al.*, *Diabetologia*, 27:599-600 (1984)).

The conclusion reached by Lopez, *et al.*, regarding the size of GLP-1 was confirmed by studying the molecular forms of GLP-1 found in the human pancreas (Uttenthal, L.O., *et al. J. Clin Endocrinol. Metabol.*, 61:472-479 (1985)). Their research showed that GLP-1 and GLP-2 are present in the pancreas as 37 and 34 amino acid peptides respectively.

The similarity between GLP-1 and glucagon suggested to early investigators that GLP-1 might have biological activity. Although some investigators found that GLP-1 could induce rat brain cells to synthesize cAMP (Hoosein, N.M., *et al.*, *Febs Lett.* 178:83-86 (1984)), other investigators failed to identify any physiological role for GLP-1 (Lopez, L.C., *et al. supra*). The failure to identify any physiological role for GLP-1 caused some investigators to question whether GLP-1 was in fact a hormone and whether the relatedness between glucagon and GLP-1 might be artifactual.

It has now been shown that biologically processed forms of GLP-1 have insulinotropic properties and delay gastric emptying. GLP-1(7-34) and GLP-1(7-35) are disclosed in U.S. Patent No: 5,118,666, herein incorporated by reference. GLP-1(7-37) is disclosed in U.S. Patent No: 5,120,712, herein incorporated by reference.

Variants and analogs of GLP-1 are known in the art. These variants and analogs include, for example, GLP-1(7-36), Gln^{9}-GLP-1(7-37), Thr^{16}-Lys^{18}-GLP-1(7-37), and Lys^{18}-

GLP-1(7-37). Derivatives of GLP-1 include, for example, acid addition salts, carboxylate salts, lower alkyl esters, and amides (see, e.g., WO91/11457). Generally, the various disclosed forms of GLP-1 are known to stimulate insulin secretion (insulinotropic action) and cAMP formation (see, e.g., Mojsov, S., *Int. J. Peptide Protein Research*, 40:333-343 (1992)).

More importantly, numerous investigators have demonstrated a predictable relationship between various *in vitro* laboratory experiments and mammalian, especially human, insulinotropic responses to exogenous administration of GLP-1, GLP-1(7-36) amide, and GLP-1(7-37) acid (see, e.g., Nauck, M.A., *et al.*, *Diabetologia*, 36:741-744 (1993); Gutniak, M., *et al.*, *New England J. of Medicine*, 326(20):1316-1322 (1992); Nauck, M.A., *et al.*, *J. Clin. Invest.*, 91:301-307 (1993); and Thorens, B., *et al.*, *Diabetes*, 42:1219-1225 (1993)).

The fundamental defects responsible for causing hyperglycemia in mature onset diabetes include impaired secretion of endogenous insulin and resistance to the effects of insulin by muscle and liver tissue (Galloway, J.S., *Diabetes Care*, 13:1209-1239, (1990)). The latter defect results in excess glucose production in the liver. Thus, whereas a normal individual releases glucose at the rate of approximately 2 mg/kg/minute, a patient with mature onset diabetes releases glucose at a rate exceeding 2.5 mg/kg/minute, resulting in a net excess of at least 70 grams of glucose per 24 hours.

Because there exists exceedingly high correlations between hepatic glucose production, fasting blood glucose levels, and overall metabolic control as indicated by glycohemoglobin measurements (Galloway, J.A., *supra*; and Galloway, J.A., *et al.*, *Clin. Therap.*, 12:460-472 (1990)), it is readily apparent that control of fasting blood glucose is essential for achieving overall normalization of metabolism sufficient to prevent hyperglycemic complications. Since existing insulin therapies rarely normalize hepatic glucose production without producing significant hyperinsulinemia and

hypoglycemia (Galloway, J.A., and Galloway, J.A., *et al.*, *supra*) alternative approaches for diabetic therapy are needed.

Therapy based on administration of longer acting GLP-1 analogs is one such approach. To date however, this approach has failed to deliver long term efficacious doses to individuals due in large part because the serum half-life of GLP-1(7-37)-based peptides is quite short. Therefore, the quest for alternative approaches continues.

Gene therapy offers a new avenue for treating diseases rooted in hormone deficiencies because it operates as an *in vivo* protein production and delivery system. This is an especially attractive approach since gene therapy also offers the possibility of physiologically regulating the production and secretion of proteins in response to homeostatic mediators within the body.

Gene therapy can be effected in a number of ways. Retroviral-mediated gene transfer was suggested for treating human diseases involving malfunctioning bone marrow. Anderson *et al.*, *Science* 226: 401 (1984). In addition, PCT Publication Number WO93/09222 (May 13, 1993) and U.S. Patent Number 5,399,346 (March 21, 1995) disclose the genetic alteration of primary human cells that are cultured then reintroduced into the body for the treatment of a variety of diseases.

Accordingly, the primary object of this invention is to provide a means for stimulating the expression and secretion of insulin and/or for delaying gastric emptying which results in lowering elevated serum glucose levels in individuals suffering from diabetes.

Summary of the Invention

A stable human cell line transformed with an expression vector comprising a DNA sequence encoding a protein of Formula 1 is claimed. The invention also includes a method of treating Type I or Type II diabetes in a patient

in need thereof comprising implanting the genetically engineered cells, which secrete a protein of Formula 1, into the patient.

Detailed Description of the Invention

Many heritable diseases such as diabetes result from the absence of a functional gene necessary to provide the animal with an adequate supply of a vital protein. Gene therapy functions to supply a functional gene encoding the protein using recombinant DNA or RNA vectors. Although there are a number of possible methods used to incorporate the desired DNA into the target cells, there are two general procedures employed termed ex vivo and in vivo therapy for introducing the gene therapy nucleotide sequence into the afflicted individual.

Ex vivo therapy consists of four primary steps: (1) Primary cells (target cells) are removed from the individual in need of therapy; (2) The gene therapy nucleotide sequence is incorporated into the target cell in vitro; (3) Transformed cells expressing the protein of interest are identified, isolated, and expanded; and (4) The transformed cells are reintroduced into the individual. Ex vivo therapy generally results in the incorporation of the nucleotide sequence encoding the protein of interest into the chromosomal DNA of the target cell. The critical step of ex vivo gene therapy is the proper introduction of the nucleotide sequence encoding the desired protein into the target cell. The introduction of the desired gene is generally accomplished by the use of viral vectors, although other methods are also applicable. Two primary types of viral vectors are frequently used for the introduction of the foreign gene into the target cell: (1) retroviral vectors and (2) adeno-associated viruses (AAVs). The choice of an appropriate retroviral vector is dependent on the existence of an appropriate viral receptor on the target cell. A variety of retroviral vectors appropriate for the

transduction of a variety of cell lines have been described in the literature. The use of retroviral vectors is generally accomplished by the use of "packaging cells" which permit the production of high titers of replication-defective recombinant virus free of wild-type virus. Miller, *Human Gene Therapy*, vol. 1, number 5 (1990). Adeno-associated viruses (AAVs) may also be used to efficiently transfer genes in vitro. Production of high titers of replication-defective recombinant virus has been described. Flotte et al. *Gene Therapy* 2:29 (1995); *Current Topics in Microbiol. Immunol.* 158:92 (1992); Kotin *PNAS* (USA) 87:2211 (1990).

In vivo gene therapy generally describes the introduction of a nucleotide sequence into an individual in need of therapy without first isolating primary cells and manipulating them in vitro. In vivo therapy generally results in foreign nucleotide sequences being transferred to the nucleus of cells of an animal without subsequent integration into the genome. Adenoviral vectors are frequently used to deliver DNA into the cells because these viruses are capable of infecting non-dividing cells and expressing foreign genes. Replication defective adenoviruses lacking portions of the E1 region of the viral genome can be propagated by growth in cells engineered to express the E1 genes. Shenk, *Cell* 16:683 (1979). Subsequent studies have indicated that replication defective adenoviruses carrying foreign DNA sequences could infect non dividing cells. Once inside the cells, the foreign DNA has been shown to express active protein. Becker et al., "Protein Expression in Animal Cells" Methods of Enzymology" (1995).

The present invention provides a method of treating both Type I and Type II diabetics through a gene therapy approach to allow the body to produce a GLP-1(7-37)-based protein. The expressed GLP-1(7-37)-based protein, in conjunction with high serum glucose levels, causes pancreatic beta cells to produce insulin in non-insulin dependent *diabetes mellitus* (NIDDM) patients and delays gastric emptying in both NIDDM and insulin dependent *diabetes*

mellitus IDDM patients. The instant invention provides gene therapy methods and constructions allowing for the in vivo production of a GLP-1(7-37)-based compound of the Formula 1:

His-Xaa^1-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Xaa^2-Gly-Gln-Ala-Ala-Xaa^3-Xaa^4-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Xaa^5

wherein

Xaa^1 is Ala, Gly, Val, Thr, and Ile; preferably Ala;

Xaa^2 is Glu, Gln, Ala, Thr, Ser, and Gly; preferably Glu;

Xaa^3 is Lys and Arg; preferably Lys;

Xaa^4 is Glu, Gln, Ala, Thr, Ser, and Gly; preferably Glu and ,

Xaa^5 is Gly-OH or is absent; preferrably Gly-OH.

Nucleotide sequences encoding any one of the compounds of Formula 1 may be prepared by a variety of means readily apparent to those skilled in the art. Wholly synthetic nucleotide sequences or semi-synthetic sequences derived in part from a natural GLP-1 gene may be used. Owing to the natural degeneracy of the genetic code, the skilled artisan will recognize that a sizable yet definite number of nucleotide sequences may be constructed which encode the compounds of the Formula 1. A synthetic DNA sequence encoding a GLP-1-based protein of Formula 1 may be prepared by techniques well known in the art in substantial accordance with the teachings of Brown, et al. (1979) *Methods in Enzymology*, Academic Press, N.Y., Vol. 68, pgs. 109-151. The DNA sequence may be generated using conventional DNA synthesizing apparatus such as an Applied Biosystems Model 380A or 380B DNA synthesizer (commercially available from Applied Biosystems, Foster City, California). Commercial services are also available for the construction of such nucleotide sequences based on the amino acid sequence.

In the preferred practice of the invention as exemplified herein a preferred synthetic coding region is assembled comprising a DNA sequence encoding a compound of the Formula 1 having the DNA sequence:

5' - CAT GCT GAA GGG ACC TTT ACC AGT GAT GTA AGT TCT TAT TTG GAA GGC CAA GCT GCC AAG GAA TTC ATT GCT TGG CTG GTG AAA GGC CGA GGA - 3'

Coding regions for Formula 1 a preferrably fused to a leader sequence which signals the cell to secrete a precursor peptide of Formula 1 that is subsequently processed by the cell to a protein of Formula. Many such leader sequences are know in the art. One well known preferred leader sequence is the hybrid tissue plasminogen activator:protein C prepropeptide described in Berg *et al.* *Biochem. Biophys. Res. Commun.* 179: 1289-1296 (1991). Typically nucleotide sequences encoding precursor peptides of Formula 1 are flanked by linker DNA to facilitate enzymatic ligation into expression vectors.

Once a suitable coding sequence of Formula 1 is constructed and optionally fused and flanked by an appropriate leader sequence and linker DNA, the construct is integrated into an expression vector then transfected into an appropriate target cell. In gene therapy, there are alternative means for introducing the genetic information necessary into a target cell.

The use of retroviral vectors allows efficient introduction of genetic information into large numbers of cells. Retroviral vectors, especially those derived from the Moloney murine leukemia virus (M-MLV), are particularly useful in the integration of foreign genetic material into the host chromosome. In the construction of such vectors, the nucleotide sequence encoding the desired compound of Formula 1 is inserted with its control region into the region of the retroviral structural gene region of the retrovirus (e.g. the gag, pol, and env genes).

Retroviral regulatory elements (enhancer and promoters) are capable of conducting efficient expression of the transduced genes in a wide variety of target cell types. However, additional regulatory sequences may be added to achieve tissue specific expression by the use of tissue specific enhancer and promoter elements.

Alternatively adenoviral vectors may be employed. Jones and Shenk (1979) Cell 16:683, demonstrated that replication deficient adenoviruses containing deletions of the E1 region of the viral genome could be propagated in cells engineered to express the E1 genes. Typical constructions of adenoviral gene therapy vectors are constructed by insertion of the foreign DNA into the E1A-E1B and E3 regions of the viral genome. Expression of the foreign DNA sequence is generally under control of the E1A promoter, the major late promoter (MLP) and associated sequences, the E3 promoter.

Other viral vectors are known for their utility in the introduction of foreign nucleotide sequences for gene therapy applications. For example, derivatives of adeno-associated virus (AAV), herpes virus and vaccinia virus vectors have been demonstrated to have utility in gene therapy.

As an alternative to viral introduction, it is possible to use receptor-mediated methods of gene transfer. In such instances, a complex is formed between the target tissue receptor and the plasmid DNA and polypeptides and the receptor on the cell surface. The plasmid DNA is taken up by the cell in endocytotic mediated transfer. The plasmid DNA may then be released from the vesicle after incorporation into the cell. Other techniques known in the art for incorporating plasmid DNA directly into target tissue rely on rapid $CaPO_4$ methodologies. See Wang *et al.*, *J. Clin. Investig.* 95 1710-1716 (1995); Ma *et al.*, *J. Biol. Chem.* 270 491-495 (1995).

Construction of suitable vectors containing the desired coding and control sequences may be constructed by

standard ligation techniques. Isolated plasmids or nucleotide fragments are cleaved, tailored, and religated in the manner necessary to achieve the desired plasmids. To effect the expression of a compounds of Formula 1, one inserts a nucleotide sequence encoding the compound into an appropriate recombinant nucleotide expression vector through the use of appropriate restriction endonucleases. The nucleotide sequence encoding a compound of Formula 1 is designed to possess restriction endonuclease cleavage sites at either end of the DNA to facilitate isolation from and integration into these amplification and expression plasmids. The coding sequence may be readily modified by the use of synthetic linkers to facilitate the incorporation of the coding sequence into the desired cloning vectors by techniques well known in the art. The particular endonucleases employed will be dictated by the restriction endonuclease cleavage pattern of the parent expression vector to be employed. The choice of restriction sites are chosen so as to properly orient the coding sequence such that it is properly associated with the promoter and ribosome binding site of the expression vector, both of which are functional in the host cell in which a compound of Formula 1 is to be expressed.

In general, plasmid vectors containing promoters and control sequences which are derived from species compatible with the host cell are used. The vector ordinarily carries a replication site as well as marker sequences which are capable of providing phenotypic selection in transformed cells.

Because the compounds of the present invention do not require post-translational processing mechanisms other than enzymatic removal of the propeptide leader sequence, many stable human cell lines may be used. One preferred cell line is the human embyonal kidney cell line 293, available from the permanent collection of the American Type Culture Collection. The techniques of transforming mammalian cells with the aforementioned vector types are well known in the

art and may be found in such general references as Maniatis, et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York or Current Protocols in Molecular Biology, Vol. 1, (1988), Wiley Interscience, and supplements.

Stable transformed cell lines that express proteins of Formula 1 must then be implanted into individuals in need thereof. The site of implantation should be compatible with the origin of the cell line used. For example, the human embryonal kidney cell line, once transformed to constitutively produce proteins of Formula 1 are preferrably transplanted under the renal capsule.

Because such cell lines generally will be histologically incompatible with the individuals receiving them, the cells are preferrably masked with $F(ab')_2$ fragments specific for HLA class I antigens. Such immunological masking methods are known in the art as described by Faust *et al. Science* 252:1700-1702 (1991). Other means for isolating the transformed cells from the recipient's immune system are consistent with this invention. Such methods include but are not limited to encasulation in semi-permeable membranes.

By way of illustration, the following examples are provided to help describe how to make and practice the various embodiments of the invention. These example are in no way meant to limit the scope of the invention.

Example 1

Construction of Intermediate
Plasmid pLP53-tLB+GLP-1

A. Preparation of BglII-Mung Bean-AvrII Digested pLP53-tLB

The plasmid pLP53-tLB was isolated from Escherichia coli K12 AG1 (on deposit under terms of the Budepest Treaty and made part of the permanent stock culture collection of the the Northern Regional Research Center,

agricultural Research Service, U.S. Dept. of Agriculture, Peoria, IL 61604 under accession number NRRL B-18714) using the Plasmid Purification Midi Kit obtained from Qiagen, Inc. (9600 DeSoto Avenue, Chatsworth, CA 91311).

Sixty µl (20 µg) of pLP53-tLB DNA was digested with 2 µl (20 units) of BglII in a 70 µl reaction volume containing 50mM Tris-HCl (pH 8.0), 10mM $MgCl_2$, and 100mM NaCl. The sample was incubated at 37°C for one hour. 17.5 µl of 5x stop mix (25% glycerol, 2% SDS, 0.05% bromophenol blue, 0.05% xylene cyanol) was added and then the reaction was heated at 70°C for 15-20 minutes to inactivate the restriction enzyme. The mixture was spin dialyzed using G-50 Sephadex Quick Spin columns obtained from Boehringer Mannheim Corporation (P.O. Box 50414, 9115 Hague Road, Indianapolis, IN 46250-0414) in order to remove the reaction components.

The 5' protruding ends created by cleavage with BglII were removed using Mung Bean Nuclease. The BglII digested pLP53-tLB DNA was incubated with 0.3 µl (3.3 units) of Mung Bean Nuclease in a 100 µl reaction volume containing 10mM Tris-HCl (pH 7.9 at 25°C), 10mM $MgCl_2$, 50mM NaCl, 1mM DTT, and 1mM $ZnSO_4$. The reaction was allowed to proceed for 30 minutes at 30°C. One µl of 1% SDS was added to inactivate the nuclease. Due to an incomplete BglII digest, the digested and undigested DNA was separated by gel electrophoresis. Ten µl of gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water) was added to the reaction. The reaction was loaded into the preparative well of a 1.5% NuSieve GTG agarose (FMC Bioproducts, 191 Thomaston Street, Rockland, ME 04841)/TAE buffer gel and then electrophoresed. The gel was stained with ethidium bromide and then the DNA was visualized by ultraviolet light. The digested DNA band was excised with a scalpel and placed into two micro-tubes. The DNA was purified from the low melting point agarose using the Wizard PCR Preps DNA Purification System obtained from Promega (2800 Woods Hollow Road, Madison, WI 53711-5399).

One hundred µl of BglII-Mung Bean digested pLP53-tLB DNA was further digested with 4 µl (16 units) of AvrII in a reaction volume of 120 µl containing 10mM Tris-HCl (pH 7.9 at 25°C), 10mM $MgCl_2$, 50mM NaCl, 1mM DTT. The sample was incubated at 37°C for 30 minutes. To prevent recircularization, the BglII-Mung Bean-AvrII digested pLP53-tLB DNA was dephosphorylated (removal of 5' phophate groups) by the addition of 2 µl (2 units) of calf intestinal alkaline phosphatase to the reaction. The sample was incubated at 37°C for an additional 30 minutes. Twenty-four µl of 5x stop mix was added. The sample was heated at 70°C for 15-20 minutes to inactivate the enzymes and then spin dialyzed using G-50 Sephadex Quick Spin columns obtained from Boehringer Mannheim Corporation (P.O. Box 50414, 9115 Hague Road, Indianapolis, IN 46250-0414) in order to remove the AvrII produced small DNA fragments. The DNA was precipitated by addition of 0.1 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol. This mixture was mixed thoroughly and then chilled at -20°C. The precipitate was collected by centrifugation for 30 minutes. The supernatant was discarded and the pellet was washed with 700 µl of cold 70% ethanol. The sample was centrifuged for 15 minutes. The supernatant was discarded, the DNA pellet was dried and then resuspended in 25 µl of water.

B. Preparation of GLP-1 Linker

The following single stranded DNA segments were conventionally synthesized by methods well known in the art on an automated DNA synthesizer (model 394 Applied Biosystems 850 Lincoln Center Drive, Foster City, CA 94404-1128) using β-cyanoethyl phosphoramidite chemistry.

GLP-1.1

5' - GACATGCTGA AGGGACCTTT ACCAGTGATG TAAGTTCTTA TTTGGAAGGC
CAAGCTGCCA AGGAATTCAT TGCTTGGCTG GTGAAAGGCC GAGGATAGGG ATCCC
- 3'

GLP-1.2

5' - CTAGGGGATC CCTATCCTCG GCCTTTCACC AGCCAAGCAA TGAATTCCTT GGCAGCTTGG CCTTCCAAAT AAGAACTTAC ATCACTGGTA AAGGTCCCTT CAGCATGTC - 3'

GLP-1.1 and GLP-1.2 are complementary DNA molecules. The synthetic DNA molecules were dissolved in water and stored at less than 0°C.

To anneal the DNA strands, 92.7 pmoles each of GLP-1.1 and GLP-1.2 were mixed in 50mM Tris-HCl (pH 7.4) and 10mM $MgCl_2$ in a total volume of 80 µl and boiled for 5 minutes. The mixture was slowly brought to room temperature and then transferred to 4°C overnight. This processed allowed the two complementary strands to anneal and form the double stranded DNA linker known as GLP-1. The linker was stored at -20°C. In order to be able to ligate into the dephosphorylated BglII-Mung Bean-AvrII digested pLP53-tLB DNA segment, the GLP-1 linker must have 5' phophate groups. The phosphate groups were added by the use of the enzyme T4 polynucleotide kinase. The kinase reaction contained 80 µl of the GLP-1 linker, 0.33µM ATP, 70mM Tris-HCl (pH 7.6), 10mM $MgCl_2$, 100mM KCl, 1mM ß-mercaptoethanol and 37.2 µl (372 units) of T4 polynucleotide kinase. The reaction was incubated at 37°C for 30 minutes. Sixteen µl of 500mM EDTA was added to stop the reaction. The reaction was extracted once with a mixture of phenol:chlorform:isoamyl alcohol 25:24:1) followed by an extraction with chloroform:isoamyl alcohol (24:1). One hundred µl of the aqueous layer was spin dialyzed using G-50 Sephadex Quick Spin columns obtained from Boehringer Mannheim Corporation (P.O. Box 50414, 9115 Hague Road, Indianapolis, IN 46250-0414) in order to remove the reaction components. The DNA was precipitated by addition of 0.1 volume of 3M sodium acetate (pH 5.2), 0.1 volume of 100mM $MgCl_2$ and 2.5 volumes of absolute ethanol. This mixture was mixed thoroughly and then chilled at -20°C. The precipitate was collected by centrifugation for 30 minutes. The supernatant was discarded and the pellet was washed with 700

µl of cold 70% ethanol. The sample was centrifuged for 15 minutes. The supernatant was discarded, the DNA pellet was dried and then resuspended in 10 µl of water.

C. Final Construction of pLP53-tLB+GLP-1

The DNA prepared in Example 1A was ligated with linker GLP-1. Two µl of DNA prepared in Example 1A and 4 µl of GLP-1 linker were ligated in a reaction that contained 2 µl (2 units) of T4 DNA ligase, 50mM Tris-HCl (pH 7.6), 10mM $MgCl_2$, 1mM ATP, 1mM DTT, and 50% (w/v) polyethylene glycol-8000 in a total volume of 10 µl. The mixture was incubated at 16°C for 16 hours. The ligation was used to transform Escherichia coli K12 INVaF' cells as generally described below.

D. Transformation Procedure

Frozen competent Escherichia coli K12 INVaF' cells are obtained from Invitrogen (3985 B Sorrento Valley Boulevard, San Diego, CA 92121). Two µl of 0.5M ß-mercaptoethanol is added to 50 µl of thawed competent cells. About 1-2 µl of the ligation reaction is mixed with the cells. The cell-DNA mixture is incubated on ice for 30 minutes, heat-shocked at 42°C for exactly 30 seconds and then chilled on ice for 2 minutes. The cell-DNA mixture is diluted into 450µl of SOC media (2% tryptone, 0.05% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose in distilled water) and incubated at 37°C for one hour in a rotary shaker set at about 225 rpm. Aliquots of upto 200 µl are plated on TY-agar plates (1% tryptone, 0.5% yeast extract, 1% NaCl, and 1.5% agar, pH 7.4) containing 100µg/ml ampicillin and then incubated at 37°C until colonies appear.

E. DNA Isolation

Following transformation, ampicillin resistant cells were picked and inoculated into 3 ml of TY broth (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4) containing

100µg/ml ampicillin. These cultures were grown for about 16 hours at 37°C with aeration. Plasmid DNA was isolated from cultures using Wizard Minipreps obtained from Promega (2800 Woods Hollow Road, Madison, WI 53711-5399).

To obtain larger amounts of pLP53-tLB+GLP-1 plasmid DNA, large scale isolation was performed using the Plasmid Purification Midi Kit obtained from Qiagen, Inc. (9600 DeSoto Avenue, Chatsworth, CA 91311).

Example 2

Construction of pGT-h+tLB+GLP-1

A. Preparation of BclI Digested pGT-h

The plasmid pGT-h was isolated from Escherichia coli K12 GM48 (on deposit under terms of the Budepest Treaty and made part of the permanent stock culture collection of the the Northern Regional Research Center, agricultural Research Service, U.S. Dept. of Agriculture, Peoria, IL 61604 under accession number NRRL B-18592) using the Plasmid Purification Midi Kit obtained from Qiagen, Inc. (9600 DeSoto Avenue, Chatsworth, CA 91311).

Ten µg (37.5 µl) of pGT-h DNA was digested to completion with 2 µl (20 units) of BclI in a 45 µl reaction volume containing 50mM Tris-HCl (pH 8.0), 10mM $MgCl_2$, 50mM NaCl. The sample was incubated at 50°C for 1 hour. Eleven µl of 5x stop mix was added to the reaction mixture. The mixture was heated at 70°C for 15-20 minutes to inactivate the restriction enzyme and then spin dialyzed using G-50 Sephadex Quick Spin columns obtained from Boehringer Mannheim Corporation (P.O. Box 50414, 9115 Hague Road, Indianapolis, IN 46250-0414) in order to remove the reaction components. The DNA was precipitated by adding 0.1 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol. This mixture was mixed thoroughly and then chilled at -20°C. The precipitate was collected by centrifugation for 30 minutes. The supernatant was discarded and the pellet was washed with

700 µl of cold 70% ethanol. The sample was centrifuged for 15 minutes. The supernatant was discarded, the DNA pellet was dried and then resuspended in 20 µl of water.

Calf intestinal alkaline phosphatase was used to remove the 5' phosphate groups from the DNA segment in order to prevent recircularization of the pGT-h. Ten µl of the BclI digested pGT-h DNA was treated with 1 µl (1 unit) of calf intestinal alkaline phosphatase in a 15 µl reaction containing 50mM Tris-HCl (pH 8.5 at 20°C) and 0.1mM EDTA. The reaction was allowed to proceed for 45 minutes at 37°C. The phosphatase was inactivated by the addition of 1µl of 500mM EDTA and then heating at 65°C for 10 minutes. The reaction volume was increased to 100µl with water and then extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) followed by extraction with chloroform: isoamyl alcohol (24:1). The DNA was recovered from the aqueous layer by the addition of 0.1 volumes of 3M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol. The mixture was mixed thoroughly and then chilled at -20°C. The precipitate was collected by centrifugation for 30 minutes. The supernatant was discarded and the pellet was washed with 700 µl of cold 70% ethanol. The sample was centrifuged for 15 minutes. The supernatant was discarded, the DNA pellet was dried and then resuspended in 25 µl of water.

B. Preparation of pLP53-tLB+GLP-1 BamHI Fragment

Thirty-five µl (10.6 µg) of pLP53-tLB+GLP-1 DNA prepare in example 1 was digested with 0.5 µl (25 units) of BamH I in a 40 µl reaction volume containing 50mM Tris-HCl pH 8.0, 10mM $MgCl_2$, and 100mM NaCl. The reaction was allowed to proceed at 37°C for one hour. Five µl of gel loading dye was added to the reaction. The reaction was loaded into the preparative well of a 4% NuSieve GTG agarose (FMC Bioproducts, 191 Thomaston Street, Rockland, ME 04841)/TAE buffer gel. The DNA was electrophoresed for about one hour at 70 constant volts. The gel was stained with ethidium bromide and then the DNA was visualized by

ultraviolet light. The desired 213 base pair DNA band was excised using a scalpel. The DNA was purified from the low melting point agarose using Wizard PCR preps obtained from Promega (2800 Woods Hollow Road, Madison, WI 53711-5399).

C. Final Construction of pGT-h+GLP-1

The DNA prepared in Example 2A was ligated with DNA prepared in Example 2B. One µl of DNA from Example 2A and 10.5 µl of DNA from Example 2B were ligated in a reaction that contained 2 µl (2 units) of T4 DNA ligase, 50mM Tris-HCl (pH 7.6), 10mM $MgCl_2$, 1mM ATP, 1mM DTT, and 50% (w/v) polyethylene glycol-8000 in a total volume of 20 µl. The mixture was incubated at 16°C for 16 hours. The ligaton reaction was used to transform Escherichia coli K12 INVaF' as described in Example 1D. Plasmid DNA was isolated from ampicillin resistant cultures as described in Example 1E.

To obtain larger amounts of pGT-h+GLP-1 plasmid DNA for the purpose of transfection of mammalian cells, large scale isolation was performed using the alkaline lysis method. Plasmid DNA was transfected in human embyonal kidney cells using standard Calcium phosphate techniques such as those described by Wigler *et al. P.N.A.S. USA* 76 1373-1376 (1979).

Example 3

Implantation

The transformed 293 cell were cultured then surgically transplanted under the kidney capsule of 8 week old Zucker Diabetic Fatty (ZDF/Gmi™-fa/fa) male rats. Under isoflurane anesthesia, a dorsal insicion was made just posterior to the diaphram, and using a rib spreader, the kidney was exposed. Approximately 20 million transformed 293 cells, in 200 µls of Hank's buffer, were injected just under the kidney capsul using a 23 guage blunt needle. The incision was sutured and protected from chewing with would clips.

We Claim:

1. A stable human cell line transformed with an expression vector comprising a DNA sequence encoding a protein of the Formula:

His-Xaa^1-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Xaa^2-Gly-Gln-Ala-Ala-Xaa^3-Xaa^4-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Xaa^5

wherein

Xaa^1 is Ala, Gly, Val, Thr, and Ile;

Xaa^2 is Glu, Gln, Ala, Thr, Ser, and Gly;

Xaa^3 is Lys, and Arg;

Xaa^4 is Glu, Gln, Ala, Thr, Ser, and Gly; and,

Xaa^5 is Gly-OH or is absent; preferrably Gly-OH.

2. The Cell line of **Claim 1** wherein the DNA sequence is

5' - CAT GCT GAA GGG ACC TTT ACC AGT GAT GTA AGT TCT TAT TTG GAA GGC CAA GCT GCC AAG GAA TTC ATT GCT TGG CTG GTG AAA GGC CGA GGA - 3'.

3. A method of treating Type I or Type II diabetes in a patient in need thereof comprising implanting the genetically engineered cells of **Claims 1 or 2** into the patient such that a protein of Formula 1 is secreted.

4. A method of treating Type I or Type II diabetes in a patient in need thereof comprising injecting an expression vector of **Claim 1** directly into the patient such that the expression vector is incorporated into the patient's cell and secretes a protein of Formula 1

5. A stable human cell line transformed with an expression vector capable of secreting a protein of Formula 1 in response to high serum glucose levels substantially as hereinbefore described with reference to any one of the Examples.

Abstract of the Disclosure

The invention provides a gene therapy method for delivering safe and effective, long term amounts of glucagon-like insulinotropic peptide capable of treating Type I and Type II diabetes. The invention eliminates the need for subcutaneous injections and is able to provide tight glucose control.

US97/01978

The Patent Office

The Patent Office
Cardiff Road
Newport
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NP9 1RH

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PRIORITY DOCUMENT

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The Patent Office

Cardiff Road
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1. Your reference
P-9872

2. Patent application number
(The Patent Office will fill in this part)
9603847.6 23 FEB 1996

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

ELI LILLY AND COMPANY,
LILLY CORPORATE CENTER,
INDIANAPOLIS,
INDIANA 46285,
UNITED STATES OF AMERICA

Patents ADP number *(if you know it)*
428904002

If the applicant is a corporate body, give the country/state of its incorporation
STATE OF INDIANA, UNITED STATES OF AMERICA

4. Title of the invention
DIABETES THERAPY

5. Name of your agent *(if you have one)*
K. G. TAPPING

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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ERL WOOD MANOR,
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SURREY GU20 6PH

Patents ADP number *(if you know it)*
31286001

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
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Signature K G Tapping Date 20TH FEBRUARY 96

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MR. K. G. TAPPING (01276) 853443

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